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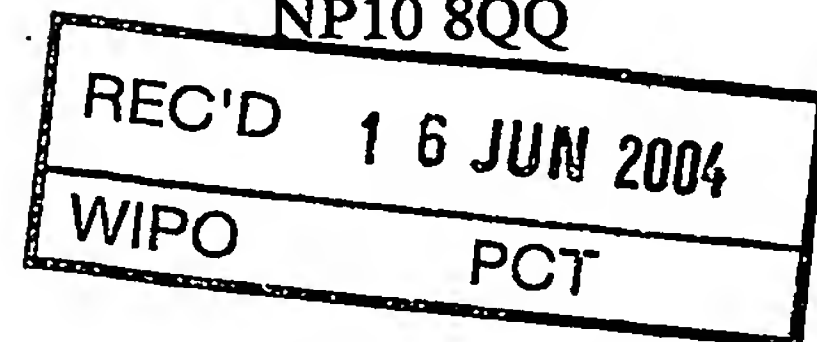


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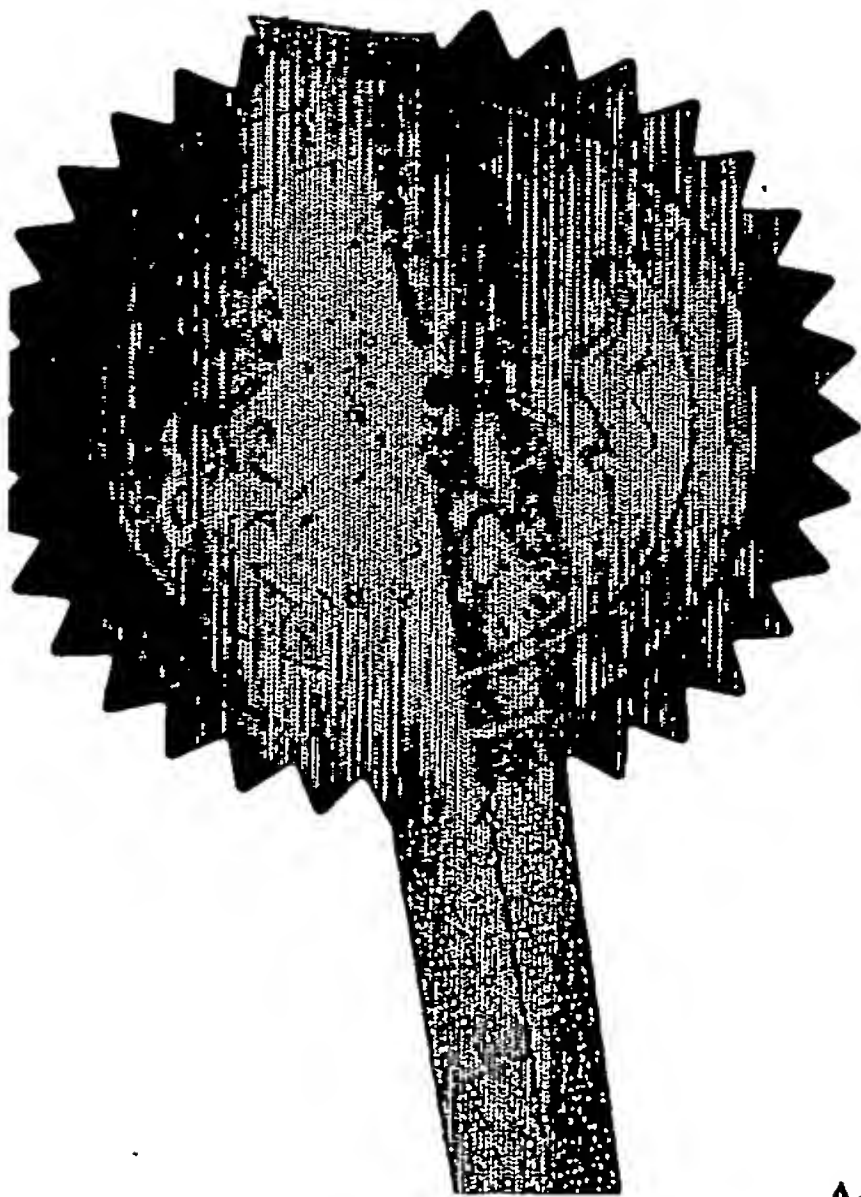


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NUCLEIC ACID DETECTION

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Nucleic Acid Detection

5 Field of the invention

This invention relates to a method for the detection of nucleic acids from biological samples. The invention also relates to a diagnostic test kit for detecting nucleic acids and primers and probes for use in the detection method.

10 Background of the invention

Severe Acute Respiratory Syndrome (SARS) is a newly identified form of atypical pneumonia in humans (Drosten et al., The New England Journal of Medicine, 10 April 2003). It is likely caused by a new strain of coronavirus (known as the SARS coronavirus or Urbani SARS-associated coronavirus). The virus is efficiently spread
15 by aerosol and person-to-person contact has been demonstrated. A diagnosis of suspected or probable SARS is made by following WHO diagnostic criteria as specified in *Case Definitions for Surveillance of Severe Acute Respiratory Syndrome (SARS)* (World Health Organization, as revised on 30 April 2003). However, a positive diagnosis of SARS cannot be made without identification of the aetiological
20 agent, either by demonstration of the presence of antibodies to the novel coronavirus or by demonstration of the presence of coronavirus nucleic acid in patient samples. The course of the disease can lead to irreversible lung damage and a mortality of 5-10% in confirmed SARS affected patients has been reported. To limit the severity of the disease it is important to identify infected patients as soon as possible in order to
25 initiate prompt treatment.

The most commonly used methods to identify viral infections are identification of antibodies in patient serum samples by standard serological methods, including but not limited to agar gel immunodiffusion (AGID) or enzyme-linked immunosorbent assay (ELISA). These methods are limited as it may take 7-21 days after infection for
30 antibodies to be produced in sufficient concentration to enable accurate and sensitive detection. Culturing the virus in susceptible cells in vitro is also a well-established method for detecting virus. However, the coronavirus associated with SARS is a novel entity and optimum growth conditions have not been described. In addition, growth of the virus requires a high level of biosecurity, as the product of the assay is

intact infectious virus. Highly skilled staff, equipment and facilities are therefore required, which limits the suitability of this technique for routine diagnostic use. In addition, the growth of the virus may be too slow to enable detection in sufficient time to initiate prompt treatment. Molecular methods, based on the amplification and detection of the virus genetic material, have previously been described for many pathogenic viruses and are applicable to the detection of the SARS coronavirus. Nucleic acid amplification methods in general are rapid, highly specific and sensitive.

Many different types of nucleic acid amplification process have been described (e.g. polymerase chain reaction (PCR) followed by agarose gel electrophoresis, nested PCR, real-time Taqman[®] PCR, molecular beacon PCR, nucleic acid sequence-based amplification (NASBA), isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN), and so forth). PCR followed by gel by electrophoresis (also known as conventional PCR) is a standard technique that was first described in 1985. It has since become a routine laboratory procedure. It is however, relatively slow and labour intensive. In addition, compared with more recent developments conventional PCR has a lower sensitivity. Nested PCR followed by gel electrophoresis is a technique that increases the sensitivity of conventional PCR. Nested PCR involves the first amplification of a specific genetic sequence using a particular pair of DNA oligonucleotide primers (outer primers). The product of this amplification is used as the template for a second round of amplification in which another pair of primers (inner primers) are selected from a region within the first amplification product. The second amplification product is thus shorter than the first amplification product. This method is very laborious as it involves an additional amplification step. Consequently, this method is easily contaminated. A highly automated method termed real-time PCR (RT-PCR) has proven to be a highly specific and user-friendly technology but occasionally may have a lower sensitivity than nested PCR. RT-PCR incorporates the use of an additional DNA oligonucleotide molecule labeled at one end with a fluorescence absorbing moiety and at the other end by a fluorescence emitting moiety, which hybridizes to a region of the target gene between the DNA oligonucleotide primers. The labelled molecule is known as a fluorogenic probe. As the amplification process continues the fluorescence signal increases due to the displacement and degradation of the fluorogenic probe by the advancing DNA polymerase enzyme. Conventional PCR, nested PR and RT-PCR alone may not be sufficiently accurate or sensitive to detect the SARS coronavirus in a wide range of patient samples. It is

therefore desirable to improve the diagnosis of SARS by detecting a specific portion of the nucleic acid of the SARS coronavirus by an amplification technique that combines high sensitivity, high specificity and speed. This is best achieved by a novel combination of standard nucleic acid amplification techniques.

- 5 It is to be understood that this invention is not limited to the detection of SARS only and is applicable to nucleic acid detection in general.

Object for the invention

- 10 It is an object of this invention to provide a highly sensitive method for detecting nucleic acids, including the SARS coronavirus, such that a person in the early stages of infection can be identified.

It is also an object of this invention to design a user-friendly diagnostic kit to detect the nucleic acid

Summary of the invention

- 15 The present invention provides a kit for detecting SARS coronavirus that includes: a nucleic acid replicating agent for replicating a target molecule, wherein the target molecule includes a nucleic acid sequence containing at least a portion complementary to the RNA sequence of the SARS coronavirus; and
- 20 a nucleic acid replicating and detecting agent for amplifying the replicated target molecule and detecting the replicated product during amplification. These agents include a primer set that can amplify the replicated product and a fluorogenic probe that is complementary to the amplified product.

- 25 The present invention also provides a process for the extraction and detection of the SARS coronavirus nucleic acid from various biological or environmental samples, which comprises the following steps:

- (A) Collecting a sample that is suspected to contain the SARS coronavirus.
- (B) Extracting the ribonucleic acid (RNA) from the collected sample
- 30 (C) Amplifying specific target sequences using RNA/DNA amplification technology
- (D) Detecting the target amplified nucleic acid products using DNA amplification technology and a suitable reporting system such as fluorogenic probes, including Taqman[®] probes or molecular beacons.

According to a first aspect of the invention, there is provided an isolated DNA sequence comprising any of SEQ ID Nos 1 to 15.

5 According to a second aspect there is provided an isolated DNA sequence comprising any of SEQ ID Nos 1 to 15 for use in DNA amplification as a primer or as a fluorescently labeled probe. The primer or probe may consist of any of SEQ ID Nos 1 to 15.

10 According to a third aspect of the invention, there is provided method for nucleic acid detection comprising the steps of nucleic acid isolation followed by DNA amplification and detection by PCR and subsequently Real Time PCR. Preferably, the method comprises the following steps:

- 15
- i) extracting the nucleic acid from a biological or environmental sample; and
 - ii) amplifying the nucleic acid using PCR; and
 - iii) amplifying and detecting the nucleic acid produced in step (ii) using Real Time PCR.

20 Still further aspects of the invention relate to an isolated DNA sequences comprising SEQ ID Nos 1 to 15 for use in a method of detecting nucleic acids and the use of the isolated DNA sequences comprising SEQ ID Nos 1 to 15 in the manufacture of a composition for use in a method of detection of nucleic acid comprising the steps:

- 25
- i) extracting the nucleic acid from a biological sample; and
 - ii) amplifying the nucleic acid using PCR; and
 - iii) amplifying and detecting the nucleic acid from step (ii) using Real Time PCR.

30 The nucleic acid to be detected is preferably derived from SARS coronavirus RNA or cDNA.

A still further aspect of the invention relates to a SARS diagnostic test kit comprising two or more isolated DNA sequences corresponding to SEQ ID Nos. 1 to 15.

Preferably, the diagnostic test kit comprises the following primer sets a) SEQ ID Nos

1,2 or 3 and 4, 5 or 6 and b) SEQ ID Nos 7,8 or 9 and 10, 11 or 12 and a probe selected from SEQ ID Nos 13, 14 or 15.

Additionally, the diagnostic test kit for detecting SARS coronavirus in a biological or environmental sample comprises:

- (i) an isolating agent for isolating the SARS coronavirus RNA from the sample; and
- (ii) a nucleic acid replicating agent for replicating a target molecule, wherein the target molecule includes: a nucleic acid sequence complementary to at least a portion of the RNA sequence of SARS coronavirus; and
- (iii) a nucleic acid detecting agent for detecting the target molecule, wherein the nucleic acid detecting agent includes the detection molecule.

Brief description of the drawings

A preferred embodiment of this invention will now be described with reference to the following figures:

Figure 1 shows a flow chart of the overall method of the detection of SARS coronavirus according to a preferred embodiment of this invention.

Figure 2 shows the method for the isolation of SARS coronavirus and its conversion to complementary DNA (cDNA) by a preferred embodiment of this invention.

Figure 3 shows the amplification of a portion of the SARS coronavirus cDNA molecule by two DNA molecules, primers A and B, and the subsequent use of the amplified product as a template for further amplification by two DNA molecules, primers C and D, with detection and quantification effected by probe E according to a preferred embodiment of this invention.

Figures 4 shows the nucleic acid sequences corresponding to SEQ ID Nos.1-15.

Detailed description of preferred embodiments

5 The present invention provides a sensitive method for detecting nucleic acids, such as the SARS coronavirus RNA or cDNA in biological and environmental samples. Such biological samples include blood, sputum, serum, plasma, nasopharyngeal swabs, oropharyngeal swabs, urine, stools, other body fluids, and also environmental samples such as water, sewage, waste materials, and so forth.

10 PCR and RealTime PCR (RT PCR) are techniques well known in the art. RT PCR may use fluorescent quantification but is not limited to fluorescent quantification only. RT PCR encompasses, for example, the use of an additional DNA oligonucleotide molecule (referred herein as the probe) preferably labelled at one end with a fluorescence absorbing moiety and at the other end by a fluorescence emitting moiety.

15 The additional DNA oligonucleotide hybridizes to a region of the nucleic acid of interest between the DNA oligonucleotide primers. Hence, the signal generator may be a fluorogenic molecule, a molecular beacon or another molecule that can be stimulated to emit photons that can be detected and quantified in a suitable detector.

 Preferred embodiments of this invention are now described with reference to

20 the figures.

Table 1 contains the reference numerals used in the figures 1 to 3.

Table 1

Number	Item
10	Single-stranded SARS coronavirus RNA
12	Chromatography column
14	Mixture of isolated nucleic acids
16	Complementary DNA (cDNA)
18	Primer A
20	Primer B
22	Extended primer A
24	Extended primer B
26	Excess double-stranded product
28	Primer C
30	Primer D
32	Probe E
34	Excess double-stranded product

The concentration of SARS coronavirus in a biological sample, for example an oropharyngeal swab, may be very low such that detection of the presence of SARS coronavirus viral RNA may not be performed on the biological sample directly. In order to increase the number of the viral RNA molecules to a sufficient amount for detection purposes, a suitable amplification technology is required. The polymerase chain reaction (PCR) is known to be a flexible technology with particular use for the amplification of DNA. Conversion of RNA into DNA allows the PCR process to be extended to cover pathogens with a RNA genome, such as the SARS coronavirus. The amplified DNA molecules may then be detected by suitable technology. Real-time polymerase chain reaction (RT-PCR) with fluorescent quantification is a rapid, highly sensitive and highly specific method for the detection of DNA and may be applicable to detection of the SARS coronavirus nucleic acid. Results can be obtained in as little as three hours.

The SARS coronavirus contains its genetic material in the form of a single strand of ribonucleic acid (RNA, 10). The viral RNA contains the genes necessary for

its reproduction and one of the essential genes is called polymerase. This gene is approximately 2800 nucleotides in length, with the nucleotides numbered from the 5' end of the molecule.

Figure 1 shows the overall procedures for the detection of SARS coronavirus by the detection kit. As shown in Figure 1, the target SARS coronavirus viral nucleic acid molecules, which are in the form of a single strand of RNA, are firstly extracted from a biological sample. The compatible biological sample types may include blood, serum/plasma, peripheral blood mononuclear cells/peripheral blood lymphocytes (PBMC/PBL), sputum, urine, faeces, throat swabs, dermal lesion swabs, cerebrospinal fluids, cervical smears, pus samples, food matrices, and tissues from various parts of the body including brain, spleen, and liver. Other samples that have not been listed may also be applicable. An isolating agent accomplishes the nucleic acid extraction process of the detection kit of this invention.

After the target SARS coronavirus viral RNA molecules (10) are extracted from the biological sample the amount of RNA molecules in the sample may not be sufficient to be detected. Therefore, a portion of the SARS coronavirus viral RNA molecule is replicated to a target nucleic acid molecule by an appropriate amplification technique, for example, polymerase chain reaction (PCR). The target nucleic acid molecules may then be detected by suitable methods.

After the overall procedures of the detection kit of the invention described, the details of each procedure will now be discussed.

The SARS coronavirus viral RNA molecules (10) may be isolated from the biological sample by applying a suitable isolating agent to the biological sample. Preferably, a lysis agent may be applied before the isolating agent. The lysis agent, for example, a lysis buffer, is responsible for dissolving the proteins and lipids, and

denaturing the proteins in the biological sample such that these materials may be removed from the sample more easily. Furthermore, the lysis agent may also serve as a buffer for stabilising the RNA molecule for long-term storage purposes. As shown in Figure 2, the RNA molecule may be stable in the lysis buffer for up to 48 hours at room temperature and may be stored indefinitely at -70°C . The advantages for doing so is that it may not be necessary to perform the analysis at the sampling site, which may not be suitable for carrying out such processes.

An example of suitable lysis buffer may include 5M guanidine thiocyanate and Tris/HCL. Such lysis buffers are well known in the art. Lysis agents having different compositions that can still achieve the purposes of dissolving proteins and lipids, denaturing proteins, and stabilising the RNA molecules may be utilised in the detection kit of this invention.

After the lysis agent has been applied to the biological sample, the next step is the isolation of the nucleic acid molecules from the sample through the use of an isolating agent. Figure 3 describes the overall isolation procedure in the detection kit. After the lysis agent is applied to the biological sample, nucleic acids together with other unwanted components are in the form of a solution. This solution is then applied to a small chromatography column (12) that has a high affinity for nucleic acid. The non-nucleic acid components are removed from the column by washing. The nucleic acids can then be specifically eluted from the column with a suitable eluent. The nucleic acid purification process described here is well known in the art.

After the nucleic acids contained in the sample are isolated, an amplification agent may then be applied to the mixture of nucleic acids (14) such that the SARS coronavirus viral RNA molecules are converted to complementary DNA (cDNA) (16) copies by use of the enzyme reverse transcriptase. This process is well known in the

art. After conversion to cDNA, a portion of the SARS coronavirus genome is replicated for detection purposes, for example by the PCR technique. To enhance the sensitivity of the detection process, the products of the PCR reaction are used in a further amplification process, namely the RT-PCR process. Five purified and isolated DNA molecules are designed for the amplification purpose, which are termed primers A to D and probe E, respectively.

Figure 3 shows a schematic diagram for the amplification of the SARS coronavirus viral RNA by a combination of PCR and RT-PCR. As shown in the figure, the amplification process is initiated by the annealing of primers A (18) and B (20) to the target SARS coronavirus viral cDNA (16), which has been rendered into a single-stranded form by heating to 95°C. The primers A and B are designed such that they are capable of binding to the target cDNA molecule. The precise location of binding depends upon the strain of virus examined. The binding site may change after a certain period of time. The important technical feature of primers A and B is that they remain capable of binding to a portion of the SARS coronavirus viral cDNA, respectively.

Accordingly, primers A and B include a binding sequence encoding a DNA sequence complementary to at least a portion of SARS coronavirus viral cDNA. For the purpose of this invention, it is found that the region suitable for the binding of primer A to the SARS coronavirus viral cDNA is a region between nucleotides 18319 to 18338 of the polymerase gene of the SARS coronavirus, which is found to contain the least number of nucleotides for the binding function. The type organism used in these studies is SARS coronavirus HKU-39849, GenBank Accession number AY278491. Therefore, the binding sequence of primer A preferably includes a DNA sequence that is complementary to region between nucleotide 18319 to 18338 of the

polymerase gene of the SARS coronavirus, which is set forth in SEQ ID No. 1 in Figure 4. It should be noted that SEQ ID No. 1 is formally written in the 5'-3' direction. As a result, the orientation of binding with respect to the viral gene is from "back" to "front".

5 As an alternative, nucleotides 18363 to 18382 (SEQ ID No.2, Figure 5) or nucleotides 18404 to 18386 (SEQ ID No.3, Figure 6) of the polymerase gene of SARS coronavirus may be used for the binding purpose in primer A.

For the purpose of this invention, it is found that the region suitable for the binding of primer B to the SARS coronavirus viral cDNA is a region between
10 nucleotides 18157 to 18179 of the polymerase gene of the SARS coronavirus, which is found to contain the least number of nucleotides for the binding function. The type organism used in these studies is SARS coronavirus HKU-39849, GenBank Accession number AY278491. Therefore, the binding sequence of primer B preferably includes a DNA sequence that is complementary to region between
15 nucleotide 18157 to 18179 of the polymerase gene of the SARS coronavirus, which is set forth in SEQ ID No. 4 in Figure 7. It should be noted that SEQ ID No. 4 is formally written in the 5'-3' direction.

As an alternative, nucleotides 18125 to 18144 (SEQ ID No.5, Figure 8) or nucleotides 18103 to 18122 (SEQ ID No.6, Figure 9) of the polymerase gene of
20 SARS coronavirus may be used for the binding purpose in primer B.

After the primers A and B bind to the SARS coronavirus viral cDNA, the primers A and B are extended through the action of a suitable polymerase, for example *Taq* DNA polymerase, in the presence of suitable nucleotides at the 3' end of primers A and B. Therefore, an extended primer A (22) and B (24) including the
25 following sequences results:

(a) a DNA sequence that is complementary to a portion of SARS coronavirus viral RNA.

By application of a suitable amplification program the SARS coronavirus cDNA can be converted into an excess of double-stranded DNA product (26). A
5 suitable amplification program is:

Initial Step	50°C, 2 min
DNA Polymerase Activation	95°C, 3-5 min
40 Cycles	95°C, 30 sec
	50°C, 45 sec
	72°C, 15 sec

To achieve the detection purpose, the amplified double-stranded DNA product (26) of the first PCR is used as the template for a further amplification utilizing RT-PCR, preferably Taqman[®] quantitative real-time polymerase chain reaction. Before it
10 can be used as the template for further amplification the replicated DNA must first be diluted in a suitable liquid in order to maximize the efficiency of the subsequent amplification process. Suitable diluents for the replicated DNA molecule include nuclease-free water or buffer. A suitable amplification program for the RT-PCR process is:

Initial Step	50°C, 2 min
DNA Polymerase Activation	95°C, 10 min
50 Cycles	95°C, 15 sec
	72°C, 1min

15

The degree of amplification can be monitored automatically in suitable equipment, e.g. ABI Prism 7700 genetic detection system.

Amplification by RT-PCR requires two DNA oligonucleotide primers (primer C (28) and primer D (30)) and a fluorogenic probe (E, 32).

For the purpose of this invention, it is found that the region suitable for the

binding of primer C to the SARS coronavirus viral cDNA amplification product is a region between nucleotides 18264 to 18245 of the polymerase gene of the SARS coronavirus, which is found to contain the least number of nucleotides for the binding function. The type organism used in these studies is SARS coronavirus HKU-39849, GenBank Accession number AY278491. Therefore, the binding sequence of primer C preferably includes a DNA sequence that is complementary to region between nucleotide 18264 to 18245 of the polymerase gene of the SARS coronavirus, which is set forth in SEQ ID No. 7 in Figure 10. It should be noted that SEQ ID No. 7 is formally written in the 5'-3' direction. As a result, the orientation of binding with respect to the viral gene is from "back" to "front".

As an alternative, nucleotides 18295 to 18314 (SEQ ID No. 8, Figure 11) or nucleotides 18322 to 18304 (SEQ ID No. 9, Figure 12) of the polymerase gene of SARS coronavirus may be used for the binding purpose in primer C.

For the purpose of this invention, it is found that the region suitable for the binding of primer D to the SARS coronavirus viral cDNA amplification product is a region between nucleotides 18193 to 18211 of the polymerase gene of the SARS coronavirus, which is found to contain the least number of nucleotides for the binding function. The type organism used in these studies is SARS coronavirus HKU-39849, GenBank Accession number AY278491. Therefore, the binding sequence of primer D preferably includes a DNA sequence that is complementary to region between nucleotide 18193 to 18211 of the polymerase gene of the SARS coronavirus, which is set forth in SEQ ID No. 10 in Figure 13. It should be noted that SEQ ID No. 10 is formally written in the 5'-3' direction.

As an alternative, nucleotides 18219 to 18336 (SEQ ID No. 11, Figure 14) or nucleotides 18205 to 18220 (SEQ ID No. 12, Figure 15) of the polymerase gene of

SARS coronavirus may be used for the binding purpose in primer D.

For the purpose of this invention, it is found that the region suitable for the binding of probe E to the SARS coronavirus viral cDNA amplification product is a region between nucleotides 18217 to 18234 of the polymerase gene of the SARS coronavirus, which is found to contain the least number of nucleotides for the binding function. The type organism used in these studies is SARS coronavirus HKU-39849, GenBank Accession number AY278491. Therefore, the binding sequence of probe E preferably includes a DNA sequence that is complementary to region between nucleotide 18217 to 18234 of the polymerase gene of the SARS coronavirus, which is set forth in SEQ ID No. 13 in Figure 16.

As an alternative, nucleotides 18241 to 18260 (SEQ ID No. 14, Figure 17) or nucleotides 18228 to 18246 (SEQ ID No. 15, Figure 18) of the polymerase gene of SARS coronavirus may be used for the binding purpose in probe E.

The primers C and D extend from the 3' end via the action of a suitable enzyme, e.g. *Taq* DNA polymerase and added nucleotides to form a double-stranded product (34). During the amplification, the probe E is displaced and degraded by the exonuclease action of the DNA polymerase enzyme. This results in an increase in the fluorescence signal which can be correlated to the degree of amplification and hence to the concentration of amplified product. The product of the RT-PCR amplification process is thus a large quantity of target DNA molecules each containing the following DNA sequences:

- (a) a DNA sequence complementary to a portion of the original SARS coronavirus

The sequence of the Taqman[®] probe may be complementary to any region of the amplified DNA product whose ends are defined by primers C and D. However, the

probe sequence cannot overlap that of the primers C and D, as this would prevent the amplification reaction from occurring.

The present invention also relates to the use of a first and a second purified and
5 isolated DNA molecules in the manufacture of a kit for the detection of SARS
coronavirus in a biological sample, wherein the RNA molecule of SARS coronavirus
is isolated from the biological sample by an isolating agent; a target molecule is
replicated by a nucleic acid replicating agent including the first and the second
purified and isolated DNA molecules, wherein the target molecule includes:
10 a nucleic acid sequence complementary to at least a portion of the RNA sequence of
SARS coronavirus; and a nucleic acid sequence for binding to a detection molecule;
the target molecule is detected by a nucleic acid detecting agent, wherein the nucleic
acid detecting agent includes the detection molecule.

15 Preferably, the target molecule is a cDNA molecule. The first purified and isolated
DNA molecule may include a DNA sequence for binding to at least a portion of the
RNA sequence of SARS coronavirus such that the first purified and isolated DNA
molecule extends in the presence of an enzyme and DNA nucleotides to generate a
DNA sequence including a DNA sequence complementary to at least a portion of the
20 RNA sequence of SARS coronavirus when the first purified and isolated DNA
molecule binds to at least a portion of the cDNA sequence of SARS coronavirus.

More preferably, the first DNA sequence may encode either one of the DNA
sequences set forth in SEQ ID Nos. 1, 2, or 3 in conjunction with either one of the
25 DNA sequences set forth in ~~SEQ ID Nos. 4, 5, or 6.~~

Additionally, the nucleic acid replicating agent may includes a second purified and isolated DNA molecule including a DNA sequence complementary to at least a portion of the RNA sequence of SARS coronavirus such that the second purified and isolated DNA molecule extends in the presence of an enzyme and DNA nucleotides to generate a DNA sequence including a DNA sequence encoding at least a portion of the RNA sequence of SARS coronavirus when the second purified and isolated DNA molecule binds to a DNA molecule including a DNA sequence complementary to at least a portion of the RNA sequence of SARS coronavirus.

10

More preferably, the DNA sequence encodes either one of the DNA sequences set forth in SEQ ID Nos. 7, 8, or 9 in conjunction with either one of the DNA sequences set forth in SEQ ID Nos. 10, 11 or 12. The replicated DNA product may be first diluted prior to the further amplification.

15

The detection molecule may includes a DNA sequence encoding at least a portion of the RNA sequence of SARS coronavirus for binding to the target molecule; and a signal generator

20 The signal generator may comprises one of the following: fluorogenic molecule, molecular beacon, another molecule that can be stimulated to emit photons that can be detected and quantified in a suitable detector. Preferably, the DNA sequence of the detection molecule encodes either one of the DNA sequences set forth in SEQ ID Nos. 13, 14 or 15.

25

The present invention is now illustrated by the following non-limiting examples. It should be noted that various changes and modifications can be applied to the following example and processes without departing from the scope of this invention. Therefore, it should be noted that the following example should be interpreted as

5 illustrative only and not limiting in any sense.

Example

10 The detailed components of the detection kit of this example are listed as follows:

Box A (Nucleic acid isolation)

Qiagen RNeasy[®] Mini Kit nucleic acid isolation kit

15

Box B (Nucleic acid amplification)

Mastermix (0.5 ml)

20 Contains dNTPs with dUTP, HotStart *Taq* DNA polymerase, MgCl₂, Uracil-N-glycosylase (UNG), and Passive Reference. UNG and dUTP work together to prevent the reamplification of carryover PCR products. UNG hydrolyses the uracil-glycosidic bonds at dU-containing DNA sites.

SARS primer (20 µl)

25 A DNA oligonucleotide complementary to a region of the polymerase gene of the coronavirus associated with SARS

SARS probe (10 µl)

30 A DNA oligonucleotide complementary to a region of the polymerase gene of the coronavirus associated with SARS and labelled with both a fluorescence emitting label and a fluorescence quenching molecule

SARS positive control (10 µl)

A highly purified and non-infectious plasmid containing a portion of the polymerase gene of the coronavirus associated with SARS

The materials as listed above are intended to be used for 50 test reactions.

5

Preparation of reagents**Box A (Nucleic acid isolation)**

Prepare sufficient number of RNeasy mini-columns (one per sample) as are required.

10

Prepare sufficient RLT buffer (0.4 ml per sample), RW1 buffer (0.7 ml per sample) and RPE buffer (0.5 ml per sample) (all provided in the RNeasy kit) as are required and warm gently to room temperature.

15

Prepare 70% ethanol (0.4 ml per sample) and nuclease-free water (50 µl per sample) as are required and warm gently to room temperature.

Box B (Nucleic acid amplification)

20

Prepare sufficient Mastermix, SARS primer, SARS probe, purified nucleic acid template (sample) and nuclease-free water as are required (see Table 1) and warm gently to room temperature.

Nucleic acid extraction (Qiagen RNeasy[®] Mini Kit)

25

1. Mix 0.4 ml sample and 0.4 ml RLT buffer
2. Add 0.4 ml 70% ethanol into a tube
3. Add 0.6 ml of the solution mix into an RNeasy column. Close the tube gently and centrifuge for 15s at ~10,000 x g. Discard the flow-through in a 2 ml collection tube. Repeat step 3.
4. Add 0.7 ml RW1 buffer to the RNeasy column. Close the tube gently and centrifuge for 15s at ~10,000 x g to wash the column. Discard the flow-through
5. Add 0.5 ml RPE buffer to the RNeasy column and centrifuge for 15s at ~10,000 x

30

- g. Discard the flow-through. Repeat step 5.
6. Centrifuge the column for extra 2 min at $\sim 10,000 \times g$
 7. Transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 50 μ l RNase-free water directly onto the silica-gel membrane. Incubate at RT for 3 min
 - 5 8. Elute RNA by centrifuge for 1 min at $\sim 10,000 \times g$

Nucleic acid amplification

Initial Step	50°C, 2 min
DNA Polymerase Activation	95°C, 3-5 min
40 Cycles	95°C, 30 sec
	50°C, 45 sec
	72°C, 15 sec

10

Following the first round PCR a portion of the product is used as template for further amplification utilising RT-PCR.

- 15 Mix the PCR components in the proportions described in Table 2.

Table 2.

PCR Set-up

Component	Volume (μ l)
Nuclease-free water	7.8
Mastermix	10
Primer	0.8
Probe	0.4
Template (100 ng)	1
Total Volume	20

20

Program the RT-PCR equipment (ABI 7700) with the amplification profile listed in Table 3.

Table 3.

5 Real-time PCR Profile (ABI Prism 7700 Sequence Detector)

Initial Step	50°C, 2 min
DNA Polymerase Activation	95°C, 10 min
50 Cycles	95°C, 15 sec. 72°C, 1min

The results of SARS coronavirus detection are listed in the following tables.

The results are confirmed by DNA sequencing using a Perkin Elmer ABI 310 Genetic

10 Analyzer.

Comparison of different amplification methods on nucleic acid samples obtained from SARS patients

15 The results were obtained by attempting to amplify nucleic acid isolated from various patient samples. The samples were obtained from SARS patients and were provided by hospitals in Beijing, China. Each sample was tested by three different methods: conventional PCR with agarose gel electrophoresis, real-time Taqman[®] PCR, and the newly described method.

Table 4:**Results of different amplification and detection technologies**

Sample number	Type of Sample	Conventional PCR	Real Time PCR	Our Method	Clinical Diagnosis
1	White Blood Cell	NEG	NEG	POS	SARS
2	Nasal swab	NEG	NEG	POS	SARS
3	Throat swab	NEG	NEG	POS	SARS
4	Throat swab	NEG	NEG	POS	SARS
5	Serum	NEG	NEG	POS	SARS
6	Nasal throat	NEG	NEG	POS	SARS
7	Stool	NEG	NEG	POS	SARS
8	Serum	NEG	NEG	POS	SARS
9	Throat swab	NEG	NEG	POS	SARS
10	Urine	NEG	NEG	POS	SARS
11	Urine	NEG	NEG	POS	SARS
12	Nasal swab	NEG	NEG	POS	SARS
13	Stool	NEG	NEG	POS	SARS
14	Serum	NEG	POS	POS	SARS
15	Urine	POS	NEG	POS	SARS

5

Result of the sensitivity study

- 10 Coronavirus RNA was extracted and transcribed into cDNA. The cDNA was serially diluted into and tested by the three methods described in Table 4.

Table 5:
Sensitivity study

Sample Number	Dilution	Conventional PCR	Real Time PCR	Our Method
1	10e-1	POS	POS	POS
2	10e-2	POS	POS	POS
3	10e-3	POS	POS	POS
4	10e-4	POS	POS	POS
5	10e-5	NEG	POS	POS
6	10e-6	NEG	POS	POS
7	10e-7	NEG	NEG	POS
8	10e-8	NEG	NEG	POS
9	10e-10	NEG	NEG	POS
10	10e-11	NEG	NEG	NEG
11	10e-12	NEG	NEG	NEG

5 As shown in the above example, it can be realised that the detection kit may be used conveniently in various testing sites. Furthermore, the detection kit is relatively easier to use than existing methods, and may be able to provide the detection results in a shorter time - the detection results may be available within three hours if desired. As it is a DNA-based detection system, the specificity and the sensitivity may be enhanced, the detection kit described in the examples are specific to the SARS coronavirus, and the concentration of the SARS coronavirus in the sample may no longer be important, as the virus will be replicated to a target molecule for detection.

10 Although the preferred embodiment of this invention has been described in previous paragraphs, it should be apparent to one skilled in the art that modifications and alternative editions of this invention are possible, and such modifications and editions are still within the scope of this invention, which is set forth in the following claims. In addition, the embodiments of this invention shall not be interpreted restrictively by the examples or figures only.

Claims:

1. An isolated DNA sequence comprising any of SEQ ID Nos 1 to 15.
- 5 2. An isolated DNA sequence as claimed in claim 1, consisting of any of SEQ ID Nos 1 to 15.
3. An isolated DNA sequence as claimed in claim 1 or 2 for use in DNA amplification.
- 10 4. An isolated DNA sequence as claimed in claim 3 for use as a primer or a probe, wherein the probe may be fluorescently labeled
5. A method for nucleic acid detection comprising the steps of nucleic acid isolation
15 followed by DNA amplification and detection by PCR and subsequently Real Time PCR (RT PCR).
6. The method for nucleic acid detection as claimed in claim 5 wherein Real Time PCR uses fluorescently labeled probes.
- 20 7. The method as claimed in any of claims 4 to 6 wherein the nucleic acid to be detected is DNA or cDNA.
8. The method as claimed in claim 7 wherein the nucleic acid is SARS coronavirus
25 cDNA.

9. The method as claimed in any of claims 4 to 8 wherein the method comprises the following steps:
- i) extracting the nucleic acid from a biological or environmental sample;
 - 5 ii) amplifying the nucleic acid using PCR; and
 - iii) amplifying and detecting the nucleic acid produced in step (ii) using Real Time PCR.
10. The method of claim 9 wherein there is an additional step after step (i) if the
- 10 nucleic acid obtained is RNA, wherein the step comprises converting the RNA to cDNA using reverse transcriptase.
11. The method as claimed in any of claims 4 to 10 wherein primers and/or probes are used in steps (ii) and (iii).
- 15
12. The method as claimed in claim 11 for the detection of SARS coronavirus cDNA wherein the primers and/or probes bind to at least a portion of SARS coronavirus cDNA.
- 20
13. The method as claimed in claim 12 wherein the primers and probes used correspond to isolated DNA sequences comprising SEQ ID nos 1 to 15,
14. The method as claimed in claim 13 wherein the primers used in step (iii) do not overlap with the probe.

15. The method as claimed in claim 13 wherein the primer set used in step (ii) corresponds to SEQ ID Nos 1,2 or 3 and 4, 5 or 6.
16. The method as claimed in claim 13 or 14 wherein the primer set used in step (iii) corresponds to SEQ ID Nos 7,8 or 9 and 10, 11 or 12.
17. The method as claimed in claim 13 or 14 wherein the probe used in step (iii) corresponds to SEQ ID Nos 13, 14 or 15.
18. An isolated DNA sequence as claimed in claim 1 for use in a method of detecting a nucleic acid.
19. An isolated DNA sequence for use as claimed in claim 18 wherein the nucleic acid is the SARS coronavirus RNA or cDNA.
20. Use of the isolated DNA sequences as claimed in claim 1 in the manufacture of a composition for use in a method of detection of nucleic acid comprising the steps:
- iv) extracting the nucleic acid from a biological sample; and
 - v) amplifying the nucleic acid using PCR; and
 - vi) amplifying and detecting the nucleic acid from step (ii) using Real Time PCR.
21. Use as claimed in claim 20 wherein the nucleic acid is DNA or cDNA

22. Use as claimed in claim 21 wherein the nucleic acid is the SARS coronavirus
cDNA.
23. Use of a primer specific to SARS coronavirus in the manufacture of a diagnostic
5 test kit for detecting viral infections wherein the virus is SARS coronavirus and
the primers consist of SEQ ID Nos 1 to 15.
24. A SARS diagnostic test kit comprising two or more isolated DNA sequences
corresponding to SEQ ID Nos. 1 to 15.
- 10 25. A SARS diagnostic test kit as claimed in claim 24 comprising the following
primer sets
- a) SEQ ID Nos 1,2 or 3 and 4, 5 or 6; and
- b) SEQ ID Nos 7,8 or 9 and 10, 11 or 12.
- 15 26. A SARS diagnostic test kit as claimed in any of claims 24 or 25 comprising
probes corresponding to SEQ ID Nos 13, 14 or 15.
27. A diagnostic test kit for detecting SARS coronavirus in a biological or
20 environmental sample wherein the kit comprises:
- (i) an isolating agent for isolating the SARS coronavirus RNA from the
sample; and
- (ii) a nucleic acid replicating agent for replicating a target molecule,
wherein the target molecule includes: a nucleic acid sequence
25 complementary to at least a portion of the RNA sequence of SARS

coronavirus; and

- (iii) a nucleic acid detecting agent for detecting the target molecule, wherein the nucleic acid detecting agent includes the detection molecule.

5

28. A kit for detecting SARS coronavirus as claimed in Claim 27, wherein the target molecule is a cDNA molecule.

10

29. A kit for detecting SARS coronavirus as claimed in Claim 27 or 28, wherein the nucleic acid replicating agent includes a first purified and isolated DNA molecule including: a DNA sequence for binding to at least a portion of the RNA sequence of SARS coronavirus such that the first purified and isolated DNA molecule extends in the presence of an enzyme and DNA nucleotides to generate a DNA sequence including: a DNA sequence complementary to at least a portion of the cDNA sequence of SARS coronavirus when the first purified and isolated DNA molecule binds to at least a portion of the cDNA sequence of SARS coronavirus.

15

20

30. A kit for detecting SARS coronavirus as claimed in any of Claims 27 to 29, wherein the first DNA sequence encodes either one of the DNA sequences of SEQ ID Nos. 1, 2, or 3 in conjunction with either one of the DNA sequences set forth in SEQ ID Nos. 4, 5, or 6.

25

31. A kit for detecting SARS coronavirus as claimed in any of Claims 27 to 30, wherein the nucleic acid replicating agent includes a second purified and

isolated DNA molecule including: a DNA sequence for binding to at least a portion of the DNA sequence of SARS coronavirus produced by a first round of amplification such that the second purified and isolated DNA molecule extends in the presence of an enzyme and DNA nucleotides to generate a DNA sequence including: a DNA sequence complementary to at least a portion of the cDNA sequence of SARS coronavirus produced during a first round of amplification when the second purified and isolated DNA molecule binds to at least a portion of the cDNA sequence of SARS coronavirus.

10

32. A kit for detecting SARS coronavirus as claimed in any of Claims 28 to 31, wherein the second DNA sequence encodes either one of the DNA sequences set forth in SEQ ID Nos. 7, 8, or 9, in conjunction with either one of the DNA sequences set forth in SEQ ID Nos. 10, 11 or 12.

15

33. A kit for detecting SARS coronavirus as claimed in any of claims Claim 27 to 32, wherein the replicated DNA product is first diluted prior to the further amplification.

20

34. A kit for detecting SARS coronavirus as claimed in any one of Claims 27 to 33, wherein the detection molecule includes; a DNA sequence encoding at least a portion complementary to the RNA sequence of SARS coronavirus for binding to the target molecule; and a signal generator.

25

35. A kit as claimed in Claim 34 wherein the DNA sequence of the DNA molecule

does not overlap with the second DNA molecules.

36. A purified and isolated DNA molecule as claimed in Claim 34 or 35, wherein
the signal generator comprises one of the following; a fluorogenic molecule, a
5 molecular beacon, or another molecule that can be stimulated to emit photons
that can be detected and quantified in a suitable detector.

37. A kit for detecting SARS coronavirus as claimed in any of Claims 34 to 36,
wherein the detection molecule encodes one of the DNA sequences of SEQ
10 ID Nos. 13, 14 or 15.

NUCLEIC ACID DETECTIONABSTRACT

5 The present invention relates to a process for the detection of nucleic acids
from biological samples. The technique involves the amplification of the nucleic
acid of interest, after conversion to cDNA if necessary, by conventional polymerase
chain reaction (PCR) after which the amplified nucleic acid is used as the template for
10 further amplification by the real-time PCR (RT-PCR) technique. This combination of
amplification procedures increases the sensitivity and specificity of the amplification
compared with conventional amplification techniques. The invention also relates to
primers for the use in this method and a diagnostic test kit.

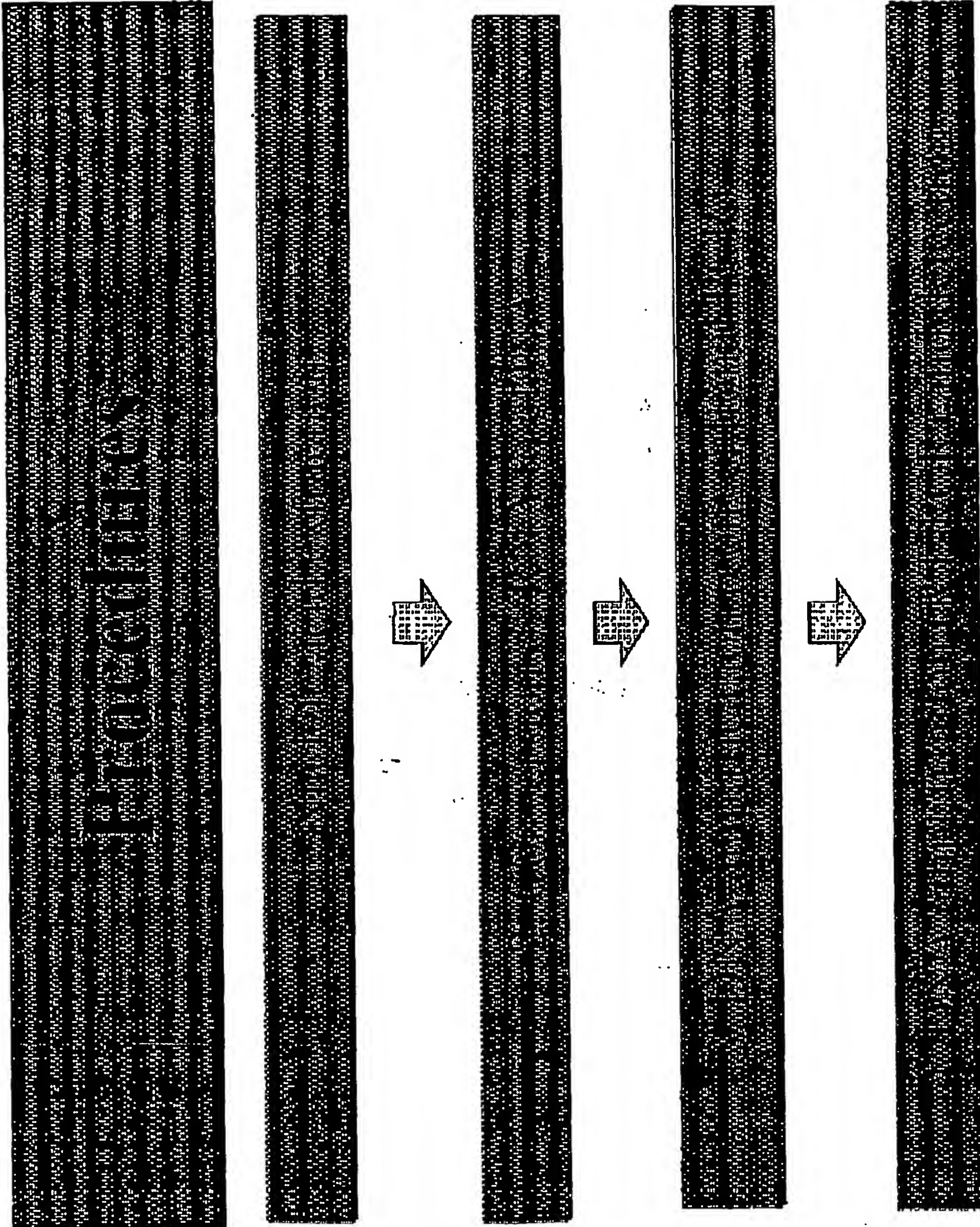


Figure 1

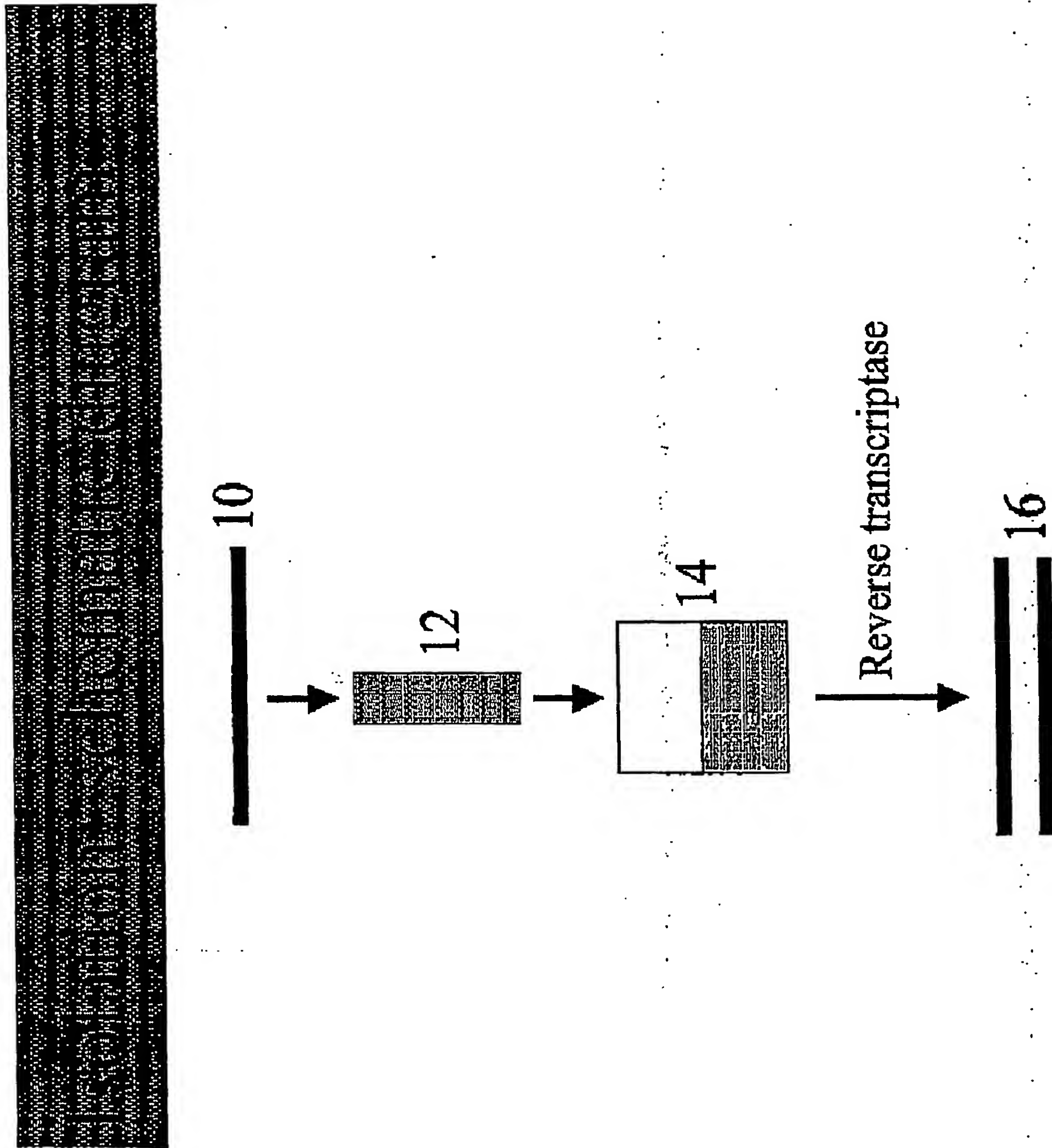


Figure 2

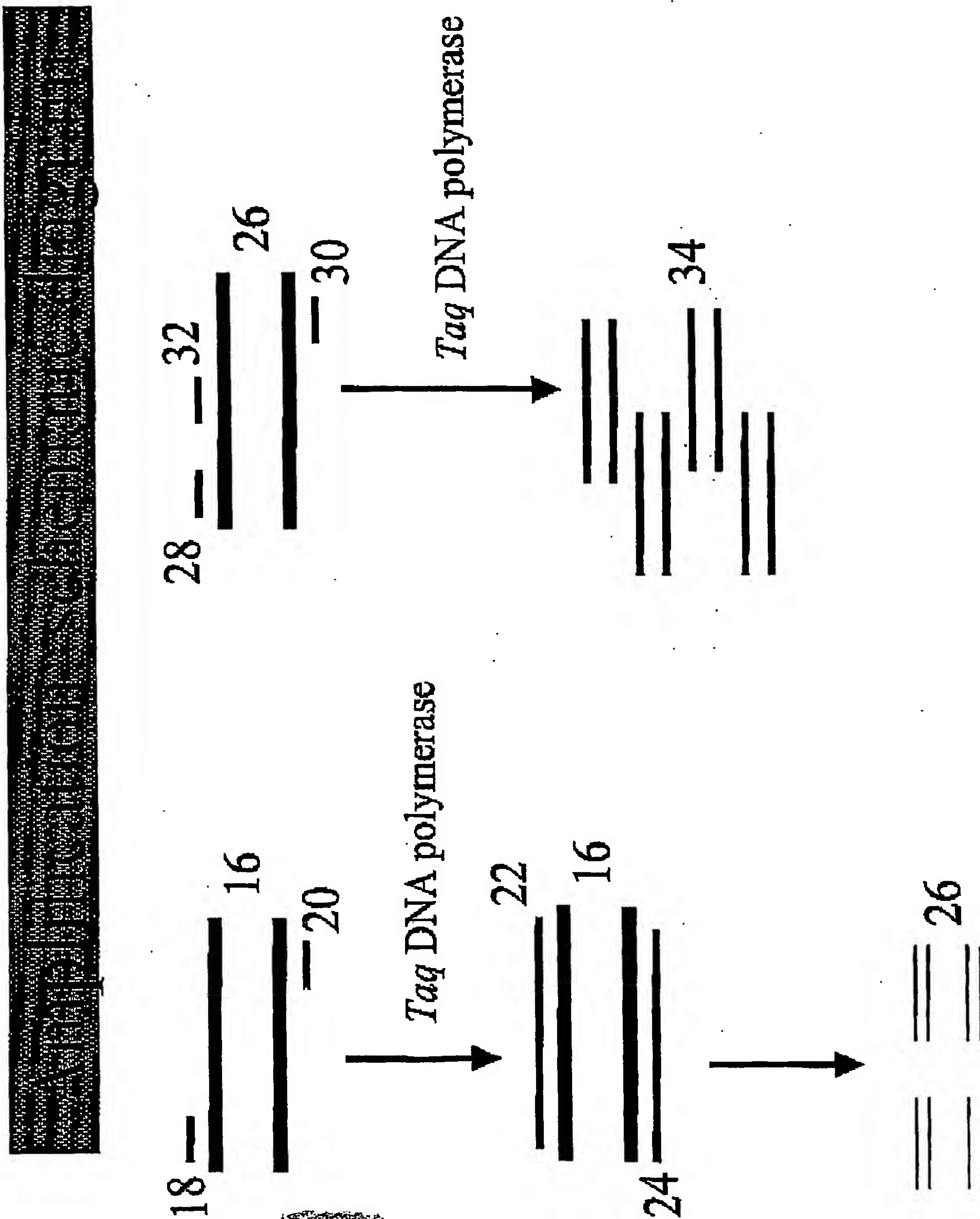


Figure 3

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Figure 4

SEQ	ID 1: ACCAGTCGGTACAGCTACTA
SEQ	ID 2: GCATTAACTCTGGTGAATTC
SEQ	ID 3: CTGGTCACCTGGTGGAGGT
SEQ	ID 4: ATTACCAAGTCAATGGTTAGGGT
SEQ	ID 5: TAGACTCATCTCTATGATGG
SEQ	ID 6: TACCAAAGGACATGACCTAC
SEQ	ID 7: CTCTAGTTGCATGACAGCCC
SEQ	ID 8: AACACCTGTAGAAAATCCTA
SEQ	ID 9: ACTAAGTTAACACCTGTAG
SEQ	ID 10: CCCGCGAAGAAGCTATTGG
SEQ	ID 11: CGTGCGTGGATTGGCTTT
SEQ	ID 12: CTATTCGTCACGTTGG
SEQ	ID 13: TTCGTGCGTGGATTGGCT
SEQ	ID 14: TAGAGGGCTGTCATGCAACT
SEQ	ID 15: ATTGGCTTTGATGTAGAGG

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